

THE DIGESTIVE SYSTEM OF THE HOLOTHURIAN, *CUCUMARIA ELONGATA*. II. DISTRIBUTION OF THE DIGESTIVE ENZYMES

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Several enzymes have been recorded from holothurian digestive tracts and in extracts of the gut wall. Oomen (1926) demonstrated a protease, amylase, maltase and a weak lipase in *Holothuria*, and Van der Heyde (1922) reported protease, invertase and lipase, but no amylase in *Thyone*. The digestive fluid and extracts of the gut wall of *Caudina chilensis* were found by Sawano (1928) to contain lipase, maltase, invertase, glycogenase and a protease resembling trypsin. Choe (1962) detected amylase, cellulase, pectinase and dipeptidase in gut extracts of both the green and red varieties of *Stichopus japonicus*. He also demonstrated small amounts of lipolytic enzyme capable of digesting simple ester, glyceride and higher fatty acid. It is now generally accepted that holothurians have abundant carbohydrate-splitting enzymes and a proteolytic enzyme similar to trypsin. Although Choe (1962) has given details of the amount of enzyme activity recorded from the different parts of the intestine of *Stichopus japonicus*, there is little information available concerning the distribution of digestive enzymes in holothurians. In the present study, extracts of the different gut regions of *Cucumaria* were tested for proteases, carbohydrases and lipases, so as to determine the distribution of enzymes as well as the enzyme complement.

It is interesting to note that Pequignat (1966) has recently demonstrated "skin digestion" in echinoids. He has shown that the mucous coating of the body surface is capable of digesting a wide range of food materials. He observed spherule coelomocytes "creeping" out to the external mucous coating where they eventually disintegrated "while probably releasing digestive enzymes." Although Pequignat has not studied holothurians, he claims that it is unlikely that they should behave any differently. However, in terms of overall nutritional requirements it is unlikely that "skin digestion" can be of serious significance to animals with well developed digestive tracts.

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MATERIALS AND METHODS

Specimens of *Cucumaria* were collected off the Northumberland coast during July and August, 1966, and the enzyme extract was prepared on the day of capture.

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Preparation of enzyme extract

Extracts were prepared of the esophagus, stomach, constriction, intestine I and intestine II. Details of gut nomenclature are given by Fish (1967).

The different regions of the gut were carefully dissected from a number of animals and placed in separate containers. After removal of the gut contents the material was quickly rinsed and then blotted dry. The gut material was weighed and added to twice its own weight of glycerol and homogenized for 10 minutes in an M.S.E. homogenizer. The volume obtained was diluted with an equal volume of filtered sea water and centrifuged for 15 minutes. The supernatant was collected and sea water of an equal volume to this supernatant was added to the residue which, after mixing, was centrifuged for a further 15 minutes. The second supernatant was added to the first to give the final enzyme extract which was filtered through a Whatman No. 4 paper. Toluene was added to prevent putrefaction. Incubation with substrate solutions was started on the day following preparation, the extracts being stored overnight at 4° C.

Estimation of proteases

Proteolytic enzymes were estimated using the formol titration method of Sørensen, as described by Davis and Smith (1955). For speed and convenience titration by indicator was preferred to the potentiometric titration recommended by Dunn and Loshakoff (1936). One-tenth per cent phenolphthalein in absolute alcohol was used as the indicator, and the enzyme-substrate mixture was titrated against approximately 0.3 N NaOH. For all titrations an "Alga" micrometer syringe was used instead of a burette. The volume of alkali delivered was accurate to 0.001 ml. The results are expressed as the amount of hydrolysis per hour per ml. of enzyme extract by using arbitrary units, *i.e.*, 0.01 ml. of 0.3 N NaOH = 10 units of hydrolysis.

The enzymes studied, together with the respective substrate solutions, were as follows:

Enzyme system	Substrate (1% aqueous solution)
Trypsin	α Benzoyl-L-arginine, ethyl ester
Aminotripeptidase	Triglycine
Glycylglycine dipeptidase	Glycylglycine
Leucine aminopeptidase	L-Leucyl-glycylglycine
Carboxypolypeptidase	Chloroacetyl-L-tyrosine

Estimation of carbohydrases

Amylase, invertase and maltase were estimated by using starch, sucrose and maltose solutions as the respective substrates.

One ml. of gut extract plus 2 ml. of substrate solution were incubated for 12 hours at 20° C. Quantitative estimations were carried out by volumetric estimation of the cuprous oxide which was formed on reduction of a cupric salt by the products of enzyme hydrolysis. The method employed was that of Bertrand, described by Plimmer (1920) with the following modifications.

Instead of boiling the reagent-sugar mixture over a bunsen flame for three minutes the mixture was heated in a boiling water bath for 15 minutes. It was

cooled and centrifuged at 3500 r.p.m. for 15 minutes, and the supernatant carefully decanted, leaving a deposit of cuprous oxide. Centrifugation is quicker and less tedious than removing the precipitate by filtration through a special asbestos filter as described in the original method. It was found necessary to avoid transference of the solution from one tube to another because the cuprous oxide becomes adsorbed onto the walls of the tube as it is being precipitated. Transference of the solution was found to result in a considerable loss of cuprous oxide. Consequently, the whole procedure, from incubation to titration, was carried out in the same tube. As a control experiment 2 ml. of substrate solution were incubated without gut extract and treated as described above, so as to account for any hydrolysis of the substrate not due to enzyme action.

The modified Bertrand method is satisfactory when dealing with the quantitative estimation of amylase, and invertase, as the substrate solutions used are not reducing sugars. However, as the method depends upon the reduction of alkaline cupric sulfate by the products of enzyme hydrolysis—reducing sugars—it is useless when testing for maltase, as the substrate used, maltose, is itself a reducing sugar. To overcome this, 10 ml. of cupric acetate solution (Barfoed's reagent) were used in place of alkaline cupric sulfate. Glucose, the product of enzyme hydrolysis, reduces Barfoed's reagent, giving a precipitate of red cuprous oxide, whereas it is unaffected by maltose.

Titration values were converted to mg. of glucose liberated by enzyme hydrolysis by reference to calibration curves obtained with glucose under conditions stated above. Although invertase on hydrolysis yields molecules of fructose as well as molecules of glucose, the reducing power of fructose is so similar to that of glucose (Plimmer, 1920), that for the purpose of constructing the calibration curves, the products of enzyme hydrolysis were regarded as molecules of glucose. Results are expressed as mg. of glucose liberated per hour per ml. of enzyme extract.

Qualitative estimation of carbohydrases

The extract used in the qualitative estimation of carbohydrases was prepared by the technique described above, using the complete gut from several animals. In each test 1 ml. of extract was incubated at 20° C. for 12 hours with 2 ml. of the respective substrate solution. Tests were made for amylase, invertase, maltase, lactase, glycogenase and cellulase.

Supplies of Laminarin and Fucoidin, substrates for cellulase activity, were obtained in the soluble powder form from the Seaweed Research Institute, Inveresk, Midlothian, Scotland.

Estimation of esterase and lipase

Esterase and lipase were estimated quantitatively by using the method of Nachlas and Seligman (1949). Beta-naphthyl esters were used as substrates. Broadly speaking, the esters of short-chain fatty acids (C_2 - C_4) are split by esterases, and the long-chain esters (C_8 -upwards) by lipases, yet according to Nachlas and Seligman (1949), there is a considerable degree of overlapping in enzymatic hydrolysis by these two enzymes. Enzymes splitting the substrate beta-naphthyl acetate (C_2) are here regarded as esterases, those splitting beta-naphthyl

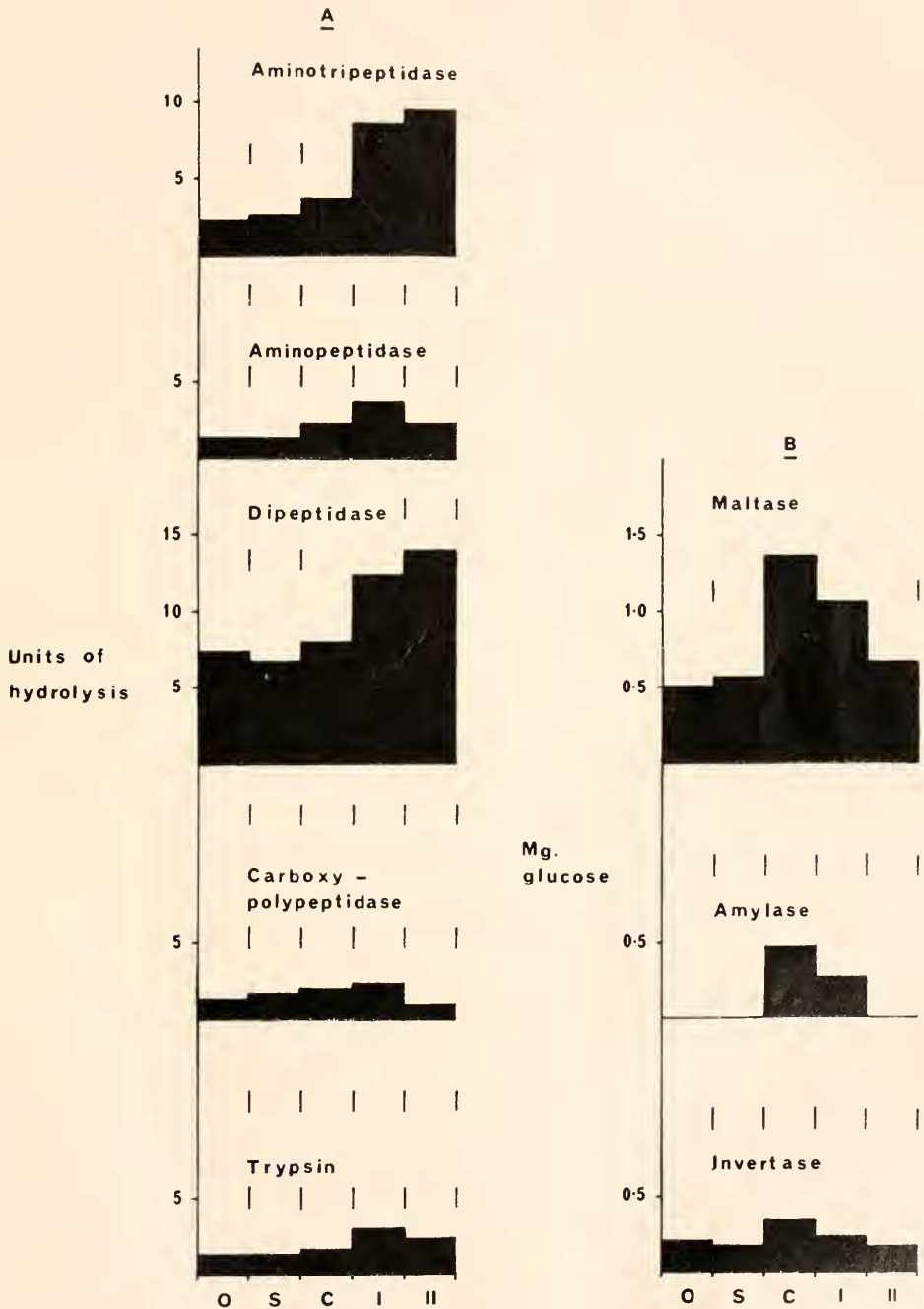


FIGURE 1. Enzyme activity recorded from the different regions of the gut. A, proteases; B, carbohydrases; O, esophagus; S, stomach; C, constriction; I, intestine I; II intestine II.

TABLE I
Activity of proteolytic enzymes from the different gut regions

	Amino-tripeptidase	Amino-peptidase	Dipeptidase	Carboxy-polypeptidase	Trypsin
Esophagus	2.2 ± 0.9	1.4 ± 0.4	7.4 ± 1.4	1.2 ± 0.5	1.3 ± 0.2
Stomach	2.6 ± 1.4	1.4 ± 1.0	6.6 ± 1.7	1.4 ± 0.4	1.2 ± 0.4
Constriction	3.7 ± 1.4	2.4 ± 1.1	8.0 ± 0.7	1.8 ± 0.8	1.6 ± 1.0
Intestine I	8.9 ± 2.1	3.8 ± 1.3	12.2 ± 2.1	2.0 ± 0.7	3.0 ± 1.3
Intestine II	9.8 ± 4.5	2.5 ± 0.3	14.0 ± 2.5	1.1 ± 0.6	2.2 ± 0.8

Activity expressed as units of hydrolysis/ml. of extract/hr. Each value is the mean and standard deviation of 5 determinations.

laurate (C_{12}) are described as "esterase-lipase" and enzymes splitting beta-naphthyl stearate (C_{18}) as lipases.

Beta-naphthol is liberated by enzymatic hydrolysis and an azo dye is produced by coupling the free naphthol with a tetrazonium salt. The colored compound was extracted with ethyl acetate and measured colorimetrically. All measurements were made using the Unicam Spectrophotometer S.P.600 at a wave-length of 540 $m\mu$. The colorimeter readings were converted to mg. of beta-naphthol by reference to a calibration curve obtained using known quantities of beta-naphthol. The results are expressed as mg. beta-naphthol liberated per hour per ml. of enzyme extract.

RESULTS AND CONCLUSIONS

Results are featured in Tables I-IV, and Figure 1, and indicate the presence of a variety of digestive enzymes in gut extracts of *Cucumaria*. An endopeptidase of a trypsin-like nature and several exopeptidases are present, and although these were detected in extracts of all gut regions, maximum activity was without exception recorded from the intestine. Maltase, amylase, and invertase were readily detected, each having maximum activity in the constriction, and glycogenase has been detected in extracts of the whole gut. Lactase and cellulase have not been detected in *Cucumaria*. The distribution of amylase is interesting, in that of the enzymes

TABLE II
Results of the qualitative estimation of carbohydrates

Enzyme	Substrate	Reagent employed	Result
Amylase	1% Starch soln.	Fehling's soln.	++
Invertase	5% Sucrose soln.	Fehling's soln.	++
Maltase	5% Maltose soln.	Barfoed's reagent	++
Lactase	2% Lactose soln.	Barfoed's reagent	-
Glycogenase	Saturated soln. of glycogen	Fehling's soln.	+
Cellulase	1% Laminarin soln.	Fehling's soln.	-
	1% Fucoidin soln.		
	1% Sodium alginate		

++ = Strongly positive; + = positive; - = negative.

TABLE III
Activity of carbohydrases from the different gut regions

	Maltase	Invertase	Amylase
Esophagus	0.51 ± 0.2	0.20 ± 0.3	0
Stomach	0.58 ± 0.2	0.17 ± 0.2	0
Constriction	1.38 ± 0.3	0.34 ± 0.2	0.49 ± 0.2
Intestine I	1.07 ± 0.2	0.25 ± 0.3	0.26 ± 0.2
Intestine II	0.66 ± 0.2	0.16 ± 0.1	0

Activity expressed as mg. reducing sugar/ml. of extract/hour. Each value is the mean and standard deviation of 5 determinations.

studied, it is the only one which is not found throughout the gut. Choe (1962) detected amylase in the first and second small intestines and the anterior and posterior parts of the large intestine of *Stichopus japonicus*.

A strong esterase activity and a weaker "esterase-lipase" have been detected with practically uniform distribution throughout the gut. The ability of the extracts to hydrolyze beta-naphthyl stearate was so poor that the results are not given. It is unlikely that the amount of enzyme activity recorded is within the limits of accuracy of the method. Oomen (1926), using amyl-acetate and ethyl butyrate esters as substrates, and Sawano (1928) using olive oil, both recorded a weak lipase in *Holothuria* and *Caudina*, respectively. It is unfortunate that there is a considerable degree of overlapping in enzymatic hydrolysis by esterases and lipases even when using purified beta-naphthyl esters as substrates. However, it can be concluded that gut extracts of *Cucumaria* hydrolyze short-chain fatty acids (C₂), and intermediate-chain fatty acids (C₁₂), yet it is doubtful whether they can hydrolyze long-chain fatty acids (C₁₈ and upwards).

DISCUSSION

Enriques (1902) suggested that digestive enzymes were carried by amoebocytes from the hemal system into the digestive tract. Oomen (1926) and Schreiber (1930, 1932a, 1932b) found that extracts of the hemal wall contained a protease, invertase, amylase and maltase, yet during his experiments Oomen found that extracts of the stomach wall contained more of these enzymes than did the hemal

TABLE IV
Activity of lipolytic enzymes from the different gut regions

	Esterase	"Esterase-lipase"
Esophagus	4.02 ± 0.4	0.26 ± 0.2
Stomach	3.03 ± 0.5	0.17 ± 0.2
Constriction	3.23 ± 0.4	0.13 ± 0.2
Intestine I	3.78 ± 0.4	0.28 ± 0.3
Intestine II	3.29 ± 0.4	0.16 ± 0.2

Activity expressed as mg. beta-naphthol/ml. of extract/hour. Each value is the mean and standard deviation of 5 determinations.

extract or the digestive fluid. However, the presence of digestive enzymes in an extract of the hemal wall was accepted as more or less verification of the hypothesis of Enriques. According to Frenzel (1892, *vide* Oomen, 1926), the walls of the rete mirabile are glandular, and their secretion is taken up by the amoebocytes and transported *via* the hemal system to the gut. The amoebocytes pass through the gut wall and between the epithelial cells into the gut lumen where they burst to release their contents. Although Hamann (1883) demonstrated gland cells in the stomachs of *Holothuria* and *Leptosynapta*, there are no records relating to the possible secretion of digestive enzymes by cells surrounding the lumen of the gut. The work of earlier authors appears to have been accepted without confirmation by detailed histochemical and physiological studies.

Studies on the digestive enzyme systems in *Cucumaria* have shown that amylase is present only in extracts of the constriction and intestine I, with a peak density in the constriction. Maltase and invertase are present in all gut regions, but there is always an obvious density peak in the constriction. Proteolytic enzymes show their greatest activity in the intestine. In *Cucumaria* there is no rete mirabile, and all parts of the hemal system have the same histological structure (Fish, 1967). If the supply of digestive enzymes is dependent upon the entry into the gut of loaded amoebocytes, then this would seem to suggest two hypotheses as regards the sites of enzyme secretion. Either the enzymes are secreted solely from the walls of the transverse branches, which are the larger channels of the system, or they are secreted in all parts of the hemal system. If the first hypothesis is valid and amoebocytes carry digestive enzymes from the transverse branches of the hemal system into the gut, then to account for the results given above (with particular reference to amylase), there must be some mechanism which ensures that amoebocytes carrying amylase pass only into the constriction and intestine I. If the second hypothesis is true and amoebocytes carry digestive enzymes from any of the hemal channels, then to account for the distribution of amylase, the dorsal and/or ventral hemal sinuses must produce enzymes needed by the particular part of the gut to which the sinus is attached. If this is the case, then the amoebocytes need only pass through the adjacent gut wall. It is suggested that neither of these hypotheses is tenable. The distribution of digestive enzymes can be correlated with the results of histological and histochemical tests applied to the different gut regions of *Cucumaria* (Fish, 1967). Secretory granules and gland cells have been demonstrated in the constriction and the intestine, respectively, and it is from extracts of these regions that the highest degrees of enzyme activity have been recorded. These results and observations lead to the conclusion that cells of the lining epithelium of the gut secrete digestive enzymes. This does not preclude the possibility that amoebocytes carry enzymes. These enzymes may play some part in the nutrition of the whole animal, or their presence may be attributed to the metabolic requirements of the amoebocytes themselves. Useful information would be gained from a detailed study of the enzyme histochemistry of the holothurian digestive tract.

SUMMARY

1. Estimations of proteases, carbohydrases, and lipases have been made on extracts of the different gut regions of *Cucumaria elongata*.

2. An endopeptidase of a trypsin-like nature and several exopeptidases have been detected, all with maximum activity in the intestine. Cellulase and lactase have not been detected, yet maltase and invertase were found in all regions of the gut, and showed their maximum activity in the constriction and intestine I. Amylase was found only in the constriction and intestine I. A strong esterase and a weaker "esterase-lipase" have been detected with practically uniform distribution throughout the gut, yet it is doubtful whether a true lipase, hydrolyzing long-chain fatty acids (C_{18} and upwards), is present.

3. The work of earlier authors dealing with digestive enzymes and the sites of enzyme production in holothurians has been summarized. Results of quantitative estimations of digestive enzymes in *Cucumaria*, coupled with the results of previous histological and histochemical studies, lead to the conclusion that digestive enzymes are secreted by cells bordering the gut lumen.

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